AMENDMENTS TO THE SPECIFICATION

The page and line numbers refer to the specification filed on October 25, 2001.

On page 40, line 21 to page 41, line 23, please amend the specification as follows, noting that the underline under the text "Figures 3A-3D" and "Figures 4A-4B" is present in the text and not indicative of new text:

Figures 3A-3D: The elution profile of the resuscitation activity. Fractions eluted from the DEAE-Sepharose™ column (see Materials and Methods) with 0.25 M KCl ~~KCl~~ were applied to a Mono Q™ column which was developed with a 20ml linear gradient from 0.08 to 0.28 M KCl ~~KCl~~ in 10 mM Tris-Cl buffer supplemented by 10% glycerol, pH 7.4. 10 ml of a diluted suspension of starved cells (CFU 3.10^{6}~~6~~cells.ml-1, total count $1.2.10^{9}$cells.ml-1) were added to 200 ml of LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract containing of 2 p1 of each fraction in 5-10 replicates in the Bioscreen™ instrument. For details see Materials and Methods. A: absorbance at 280nm and magnitude of KCl concentration. B: resuscitation activity. C: SDS-PAGE profile of the fractions following DEAE-cellulose and Mono Q™ chromatography. Lanes 1, markers (94,000, 67,000, 43,000, 30,000, 20,100, 14,400); 2, fraction from DEAE-cellulose column; 3, purified preparation (fraction number 8 from the Mono Q™ - column). D: Reduction of apparent lag phase of viable cells. 10 µl of a diluted suspension of viable, stationary phase cells (viable count 20 cells) was added to 200 ml of LMM supplemented with 0.5 % w/v L- lactate and containing 2 µl of each fraction (from a different experiment to that shown in parts A and B) in 5-10 replicates in the Bioscreen™ instrument. The apparent lag phase was estimated by extrapolating the exponential growth line to the abscissa.

Figures 4A-4B: Effect of purified RP-factor on *M. luteus*. A. Concentration dependence of RP-factor activity for resuscitation: resuscitation of dormant cells with different concentrations of RP-factor. 10µl of a diluted suspension of starved cells (CFU 3.10^{6} cells.ml-1, total count 5.10^{9} cells.ml-1) was added to 200 µ1of LMM supplemented with 0.5 % w/v L-lactate, 0.05% yeast extract and RP-factor in concentrations shown in 5-10 replicates in the Bioscreen™ instrument. For details see Materials and Methods.

B. Growth of washed cells. Stationary phase cells of ~~M. luteus~~ *M. luteus* grown in LMM were washed five times by suspension and centrifugation in LMM from which lactate had

been omitted. Bacteria were finally suspended in the same medium by repeatedly passing them through a syringe, diluted and inoculated into a 20 ml flask containing LMM or LMM plus 31 pM RP-factor. The initial cell density was 250 viable cells per ml and incubation was at 30°C with intensive shaking. Growth was monitored by plating 0.lml samples on plates containing broth E solidified with agar.

On page 42, line 18 to page 43, line 6, please amend the specification as follows, noting that the underline under the text "Figures 6A and B" and "Figures 7A-7C" is present in the text and not indicative of new text:

Figures 6A and B : Effect of *M. luteus* RP-factor on the growth of *Mycobacterium smegmatis* (A) and *Mycobacterium bovis* (B) in batch culture as observed turbidimetrically. *M. smegmatis* was grown in broth E, to which was added RP-factor at 31 pMol/L. Cells were inoculated at a level of circa 200 per well, and growth was monitored in the Bioscreen™ instrument. *M. bovis* was grown in Sauton medium, as described in the Materials and Methods section, to which RP-factor (620 pMol/L) was either added or not. The inoculum was circa 1.10^5 cells.ml-1, and the OD shown is the average of 10 separate determinations of 10 separate tubes.

Figures 7A-7C: A: Purification of His-tagged RP-factor. RP-factor was expressed in ~~E. coli~~ *E. coli* HSM174(DE3) and purified as described infra. Shown is the SDS-PAGE profile of fractions following Ni^{2+}-chelation chromatography. The molecular weight (kDal) markers (SIGMA) were bovine serum albumin (67), ovalbumin (43), glyceraldehyde 3-phosphate dehydrogenase (36), carbonic anhydrase (30), soya bean trypsin inhibitor (20.1), and lactalbumin (14.4). Lane: 1, markers; 2, crude extract from ~~E. coli~~ *E. coli* containing pET19b vector; 3, crude extract from ~~E. coli~~ *E. coli* containing pRPF1; 4, purified recombinant RP- factor.

B: Reduction of the apparent lag phase of viable cells of *M. luteus* by purified 5 recombinant RP-factor. For experimental details see the legend for Figure 3C. A dilution factor of 10^0 corresponds to 33 µg ~~ig~~ RP-factor/ml.

On page 44, lines 5-15, please amend the specification as follows, noting that the underline under the text "Figure 10" is present in the text and not indicative of new text:

Figure 10: Effect of recombinant RP-factor on growth of M. tuberculosis in Sauton medium. Sauton medium containing 0.05% Tween®-80 and 100µMol/L Na oleate + 10% (v/v) supplement (which contains, per litre, 50g bovine serum albumin, 20g glucose, 8.5g NaCl) was inoculated to an initial cell density of $31x10^3$ cfu/ml (viable count determined by plating on agar-solidified Middlebrook 7H9 medium containing 10% v/v supplement, composition as detailed above) [total count by microscopy = 10^6 cells per ml] with a 2.5 month-old culture of M. tuberculosis strain H37Ra grown in the same medium. Growth of tube cultures at 37^0C was measured by determining the OD600nm at intervals for 28 days. The undiluted concentrations of the RP-factors, Rpf (*M. luteus*) and Rpf2 (*M. tuberculosis*), employed for these experiments were ca. 10µg/ml.

On page 45, lines 9 to 14, please amend the specification as follows:

The MPN assay was performed in a Bioscreen™ C optical growth analyzer (Labsystems, Finland) using lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract as a resuscitation medium. Dilutions of starved cells were made as described. 10 µl of each dilution (5-10 replicates) were added to a well containing 200 µl of either lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract or the same medium with fraction tested (2-20 µl). Growth (optical density) was monitored using a 600 nm filter. Plates were incubated at 30°C with intensive continuous shaking. The overall measurement period was 120h, each well being measured hourly.

On page 46, line 14 to page 47, line 13, please amend the specification as follows:

Pre-wetted DEAE cellulose was added to culture supernatant (1:10 v/v) and incubated at 4°C for lh with slow stirring. The cellulose was loaded into a column, and washed with 5 volumes of buffer 1 consisting of 10mM Tris-Cl, 1mM EDTA, 1mM DTT, 10% (v/v) glycerol, pH 7.4 with 10mM KCl. The column was eluted stepwise with 2-3 bed volumes of 0.3M KCl in buffer 1. The fraction obtained was slowly diluted with buffer 1 on ice to give a final KCl concentration of 0.08M. Forty column volumes of this fraction was then loaded onto a DEAE-~~sepharose~~ Sepharose® fast flow column (1 part of sepharose pre-equilibrated with buffer 1 containing 0.08M KC1). The column was washed with 5 bed volumes buffer 1 containing 0.08M KC1 and eluted stepwise with 3 volumes of 0.25M KC1 in buffer 1. The fraction

obtained was again slowly diluted with buffer 1 on ice to a final KC1 concentration of 0.08M, filtered through a 0.22 μm Gelman filter and loaded onto a Mono Q™ column (model HR5/5, pre-packed, Pharmacia) equilibrated with buffer 2 consisting of 10mM Tris-Cl, 10% glycerol, pH 7.4 containing 0.08M KC1. The Mono Q™ column was eluted by a linear gradient from 0.08 M to 0.28 M KC1 in buffer 2 (the total volume of the elution was 20 ml). The flow rate and fraction size were 1 ml/min and 1 ml/tube respectively. All manipulations except the Mono Q™ chromatography step were performed at 4°C. The fractions obtained were dialysed against 10 mM Tris-Cl containing 10% glycerol (dialysis is important for the retention of activity) and stored at 4°C for up to 5 days without loss of activity. For prolonged storage in a deep freeze, fractions were dialysed in the same way and glycerol added to a final concentration of 20-30% w/v. The protein content in purified preparations was estimated by tryptophan fluorescence using lysozyme as a standard.

Trypsin treatment:

Trypsin was added to the active, dialysed fraction obtained from the ~~mono~~ Mono Q™ column and diluted by LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract (1:100) (the final concentration of trypsin was 50 ug/ml). The mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of trypsin inhibitor (100 ug/ml). In control experiments trypsin inhibitor was added to the mixture (100 ug/ml) prior to incubation.

On page 47, lines 25-29, please amend the specification as follows:

Nutrient Broth E, yeast extract and agar were obtained from Lab M, whilst L-lactate (Li salt), succinate, trypsin, soybean trypsin inhibitor and DEAE-Sepharose fast flow were obtained from Sigma. DEAE cellulose DE52 was obtained from Whatman, and Mono S™ and Mono Q™ from Pharmacia. Other chemicals were of analytical grade and were obtained from Sigma or BDH.

On page 49, lines 7-13, please amend the specification as follows:

Using succinate-grown cultures, the active fraction was purified by a combination of anion exchange media (see Material and Methods). The final activity was eluted at around 180 mM KC1 from a linear KC1 gradient (from 0.08 to 0.28M KC1) on a MonoQ™ column in 3

adjacent fractions (Fig. 3). It is worth mentioning that it proved important to dialyse the fractions before testing their activity because some fractions were inactive before dialysis. Active fractions did not change their resuscitation activity after dilution up to 400 times (v/v).

On page 51, lines 10-21, please amend the specification as follows:

The sequence information shows that the RP-factor gene and all of its mycobacterial homologues share a secretory signal sequence and a particularly highly conserved, ca. 70-residue segment. One (MTubZ94752) also has a membrane anchoring motif. The conserved 70-residue segment is a candidate for a signalling domain. Most of this segment is weakly hydrophilic (Kyte-Doolittle) and is predicted to form amphipathic α ~~á~~-helical (Garner-Robson; Chou-Fasman) or β-sheet regions (Eisenberg). Overall, the segment has a low surface probability (Emini). The C-terminal section, by contrast, is much less highly conserved and might be considered a better candidate for determining localization or specificity (i.e. be a cellular compartment-targeting or specificity-determining domain). By analogy with other protein signalling systems (e.g. many pro hormones in animals, and systemin in plants) it is possible that the proximate signalling molecule is a proteolytically cleaved product.

On page 53, lines 4-10, please amend the specification as follows:

[$]Growth was estimated microscopically (magnification times 600) after 14 days of 5 incubation; ca. 50 µ1 of each culture was fixed, stained using Ziehl-Neelsen reagent and counted. Values in the body of the Table are average numbers of cells in a microscope field (10-20 fields counted) ± standard deviation with the number of determinations in parentheses. RP-factor (after elution from the Mono Q™ column and dialysis) was used at a concentration of circa 40 pMol/L; activity was lost after either trypsin treatment, heating (autoclaving) or filtration through a 12 kDal cutoff membrane.

On page 54, line 21 to page 55, line 12, please amend the specification as follows, noting that the text "Analysis of recombinant RP-factor" was underlined in the original and does not indicate new text:

Two primers [5'-GTCAGAATTCATATGGCCACCGTGGACACCTGGG-3'] (SEQ ID NO: 46) and [5'-TGACGGATCCTATTAGGCCTGCGGCAGGACGAG-3'] (SEQ ID NO:

47) were employed to amplify (5 cycles of 30s at 94°C, 30s at 60°C, 30s at 72°C, followed by 15 cycles of 30s at 94°C, 60s at 72°C) the RP-factor coding sequence (i.e. lacking the signal sequence) from the cloned 1.4 kbp SmaI fragment of genomic DNA. It was first established in E. coli ~~DH5á~~ DH5α as a 567 bp *Eco*RI-*Bam*HI fragment in pMTL20 and then excised as a 562 bp *Nde*I - *Bam*HI fragment, inserted into pET19b (Novagen) and re-established in *E coli* ~~DH5á~~ DH5α . The sequence of the PCR product and vector-insert junction in this plasmid, denoted pRPF1, was verified. RP-factor was expressed from RPF1 after transforming it into *E. coli* HSM174(DE3). The protein, containing a His_{10}-tag at the N-terminus, was isolated by sonicating bacteria, previously grown to an OD_{600nm}=0.6 and induced with 0.4 mM IPTG for 4 h, in a modified binding buffer (MBB - 5mM imidazole pH7.9/0.5M NaCl/20mM Tris-HC1/8M urea) containing 5 mM DTT and 2 mM EDTA. After low speed centrifugation, low MW compounds, including EDTA and DTT, were removed by elution through a Sephadex® G10 column pre-equilibrated with MBB. A Ni^{2+}-chelation column (Ni^{2+}-coordinated iminodiacetic acid immobilized on Sepharose® 6B), was loaded with the G10 eluate, washed with 20 vol MBB and then successively eluted with four 10 vol aliquots of MBB containing 0.01 M, 0.05 M, 0.2 M and 1 M imidazole, respectively. The column was finally eluted with strip buffer (20 mM Tris-HC1, pH 7.9/100 mM EDTA/0.5 M NaCl). Monoclonal anti-(polyHis) antibodies (Sigma, clone His-1) were employed for immunoblot analysis of fractions subjected to SDS PAGE electrophoresis and electroblotted using standard methods. Fractions were dialysed against buffer 2 and assayed for biological activity as indicated above.

On page 56, lines 3-10, please amend the specification as follows:

A rabbit was immunized three times at one week intervals using recombinant RP-factor (the recombinant protein prepared as described above). The protein was administered at 300 μg of protein per injection in incomplete Freud's adjuvant (0.5 ml protein and 0.5 ml adjuvant) Blood was collected before administration was started and on the 11th day after the last injection. The immunoglobulin fraction was obtained by standard procedures using PEG. Antibodies were additionally purified on a protein G-~~superose~~ Superose™ column according to the standard (Pharmacia) protocol. The final protein concentration to was adjusted spectrophotometrically to 1 mg/ml.

On page 56, line 24 to page 57, line 14, please amend the specification as follows:

Two primers [5'-ATCAGAATTCATATGGACGACATCGATTGGGACGC-3'] (SEQ ID NO: 48) and [5'-CGCAGGATCCCCTCAATCGTCCCTGCTCC-3'] (SEQ ID NO: 49) were employed to amplify (5 cycles of 30s at 94°C, 30s at 58°C, 30s at 72°C, followed by 25 cycles of 30s at 94°C, 60s at 72°C) the RP-factor coding sequence (i.e., lacking the signal sequence) from *M. tuberculosis* H37Rv genomic DNA. The PCR product was first established in *E. coli* ~~DH5α~~ DH5α as a 336 bp *Eco*RI-*Bam*HI fragment in pMTL20 and then excised as a 331 bp *Nde*I-*Bam*HI fragment, inserted into pET19b (Novagen) and re-established in *E. coli* ~~DH5α~~ DH5α. The sequence of the PCR product and vector-insert junction in this plasmid, denoted pRPF2, was verified. The *M. tuberculosis* RP-factor was expressed from pRPF2 after transforming it into *E. coli* HSM174(DE3). The protein, containing a His10-tag at the N-terminus, was isolated by sonicating bacteria, previously grown to an OD_{600nm} = 0.9 and induced with 0.4 mM IPTG for 4 h, in binding buffer (BB - 5mM imidazole pH7.9 / 0.5M NaCl / 20 mM Tris-HCl / 8M urea). After low speed centrifugation, a Ni2+-chelation column (Ni^{2+}-coordinated iminodiacetic acid immobilised on Sepharose® 6B), was loaded with the supernatant, washed with 20 vol 13B, 20 vol BB containing 100 mM imidazole, and then eluted with 10 vol BB containing 0.5 M imidazole. Additional purification was achieved by MonoQ® column chromatography (vide infra, save that the salt gradient was from 0.1 M to 1M NaCl). Monoclonal anti-(polyHis) antibodies (Sigma, clone His-1) were employed for immunoblot analysis of fractions subjected to SDS PAGE electrophoresis and electroblotted using standard methods. Fractions were dialysed against buffer 2 and assayed for biological activity as indicated above.

On page 59, lines 14-19, please amend the specification as follows:

Macrophages were grown as a monolayer on plastic petri dishes (10^6 cells/5 cm^2) in standard RPMI medium containing gentamicin and penicillin (10 µg~~ig~~/ml, each) under standard conditions (CO_2/O_2 mixture in a 37°C incubator). M. tuberculosis cells were recovered from macrophages by passing them repeatedly through a thin syringe needle. Macrophage cell debris was removed by low speed centifugation and M. tuberculosis cells were then collected by centrifugation at higher speed.

On page 59, line 23 to page 60, line 23, please amend the specification as follows:

The entire *yabE* coding region together with flanking sequences was amplified from B. subtilis genomic DNA using primers D11 [5'-GAAGAGAATTCCTTCCATCACGA-3'] (SEQ ID NO: 50) and D12 [5'-CCAAACGAATTCGGTCAATCAC-31 (SEQ ID NO: 51) as a 1803 bp product. A 1186 bp *Hind*III-*Bcl*I fragment encompassing the 3' end of the coding sequence was excised from the PCR product, ligated with *Hin*dIII + *Bam*HI-digested pMTL20, and used to transform *E. coli* strain ~~DH5α~~ DH5α with selection for ampicillin-resistance. Plasmid pYABE was isolated from one of the transformants. A 763 bp *Hin*dIII-*Bam*HI fragment from entirely within the *yabE* coding sequence was excised from the pYABE, ligated with *Hin*dIII + *Bam*HI -digested pMUTIN4, an integrating plasmid that may be employed for generating knockout mutations in *B. subtilis* (Edwards & Errington, 1997, Molecular Microbiology, 24, 905-915) and used to transform *E. coli* strain XL 1-Blue with selection for ampicillin-resistance. Plasmid pYAB2, containing an internal segment of the *yabE* coding sequence, was isolated from one of the transformants. A 1207 bp *Hin*dIII-*Eco*RI fragment encompassing the 3' end of the yabE coding sequence was excised from pYABE, ligated with *Hin*dIII + *Eco*RI digested pMUTIN4 and used to transform *E coli* strain XL1-Blue with selection for ampicillin-resistance. Plasmid pYAB3, containing the 3' end of the *yabE* coding sequence, was isolated from one of the transformants.

The entire *yocH* coding region together with flanking sequences was amplified from *B. subtilis* genomic DNA using primers D10 [5'- GCAAGGATCCCAGACTAAAAAAACAG-3'] (SEQ ID NO: 52) and D9 [5'- ATCAGGATCCATATTATTAGTTTAAGA-3'] (SEQ ID NO: 53) as a 1145 bp product. A 358 bp *Hpa*I fragment from entirely within the *yocH* coding sequence was excised from the PCR product, ligated with SmaI-digested pMTL20, and used to transform *E. coli* strain XL1- Blue with selection for ampicillin-resistance. Plasmid pYOC2a, containing an internal segment of the *yocH* coding sequence, was isolated from one of the transformants. The insert in this plasmid was then excised from pYOC2a as a 385 bp EcoRI-*Hin*dIII fragment and inserted into pMUTIN4, to yield pYOC2. A 307 bp *Hin*dIII -*Bam*HI fragment encompassing the 3' end of the yocH coding sequence was excised from the 1145 bp PCR product, ligated with *Hin*dIII + *Bam*HI digested pMUTIN4, and used to transform E. coli strain ~~DH5α~~ DH5α with selection for ampicillin-resistance. Plasmid pYOC3, containing a DNA

segment encompassing the 3' end of the *yocH* coding sequence, was isolated from one of the transformants.